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Development of real-time diagnostic assays specific for *Mycoplasma mycoides*s subspecies *mycoides* Small Colony

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Abstract

Rapid and specific detection of *Mycoplasma mycoides* subsp. *mycoides* Small Colony (*M. mycoides* SC) is important for the effective control of contagious bovine pleuropneumonia. Although the United States has been free of this disease for over 100 years, it is necessary to develop modern diagnostic assays that are sensitive and specific for biological agents that would affect the US agricultural industry following accidental or intentional introduction into the US agricultural population. With this aim in mind, we have identified *M. mycoides* SC-specific genetic loci and developed TaqMan-based PCR assays for the detection of *M. mycoides* SC. The TaqMan assay allows for real-time detection of specific, amplified PCR products using portable equipment, enabling testing to be performed in the field. These assays are specific for *M. mycoides* SC, failing to amplify DNA from other organisms belonging to the *M. mycoides* cluster or two phylogenetically unrelated bovine mycoplasma species. Standard curves were drawn based on the linear relationships measured between the threshold fluorescence (C_T) values and a measured quantity of genomic DNA. *M. mycoides* SC was successfully detected in bronchoalveolar lavage samples obtained from experimentally infected cattle. These TaqMan-based real-time PCR assays will allow for the rapid and specific detection of *M. mycoides* SC.

Keywords: *Mycoplasma mycoides* subsp. *mycoides* Small Colony; Contagious bovine pleuropneumonia; Diagnostic; Real-time PCR

1. Introduction

Mycoplasma mycoides subspecies *mycoides* Small Colony (*M. mycoides* SC) is the etiologic agent of contagious bovine pleuropneumoniae (CBPP), a severe

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respiratory disease of cattle. *M. mycoides* Small Colony belongs to the *M. mycoides* cluster that is comprised of six species or subspecies of related mycoplasma of ruminants, including *M. mycoides* subsp. *mycoides* Large Colony (MmmLC), *M. mycoides* subsp. *capri*, *Mycoplasma capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, and *Mycoplasma* sp. bovine serogroup 7 (Cottew et al., 1987; Pettersson et al., 1996). The United States Department of Agriculture (USDA) classifies *M. mycoides* SC as a high consequence livestock pathogen and CBPP is classified as a list A disease by the International Office of Epizootics (OIE). Although the United States of America was declared free of CBPP in 1892, this disease persists in many African countries and is considered one of the most economically significant diseases of cattle in Africa (Windsor, 2000).

The complement fixation (CF) test is currently the prescribed test for the detection of *M. mycoides* SC at the herd level and is highly specific for diagnosing CBPP in animals with acute infection; however, it is less effective at the early and chronic stages of disease (Amanfu et al., 1998; Amanfu et al., 2000; OIE, 2004). In addition, the complexity of this test makes it time-consuming, expensive, difficult to accurately and consistently obtain and interpret reliable results, and not amenable for use as a ''pen-side'' or field test (OIE, 2004).

Alternative tests have been developed for the diagnosis of CBPP. A biochemical assay has been developed to identify *M. mycoides* SC based on its inability to utilize maltose, a unique characteristic among the members of the *M. mycoides* cluster, but this assay requires direct culture of the organism (Rice et al., 2000). Agglutination assays for *M. mycoides* SC are rapid and can be performed in the field; however, they have reduced sensitivity and specificity compared to many other tests (Turner and Etheridge, 1963; Adler and Etheridge, 1964; March et al., 2003). Immunoblot and ELISA techniques present issues of serological cross-reactions with related species of mycoplasmas, can be time-consuming, and require complex, non-mobile, laboratory settings (Nicholas et al., 1996; Le Goff and Thiaucourt, 1998). Various PCR assays able to detect *M. mycoides* SC nucleic acids are fast, specific, and sensitive, but those developed require post-PCR manipulations to obtain results (e.g. restriction endonuclease analysis and agarose gel electrophoresis) (Bashiruddin et al., 1994, 1999; Dedieu et al., 1994; Rawadi et al., 1995; Hotzel et al., 1996; Miserez et al., 1997; Persson et al., 1999).

To date, no single diagnostic assay is relied upon for the detection of *M. mycoides* SC. A recent study compared the use of direct culture, sandwich ELISA, and PCR and demonstrated that no single test matched the pathological findings observed with CBPP

Table 1
Mycoplasma species and strains tested with results of real-time PCR for three *M. mycoides* SC-specific TaqMan probes

Species	Strain	Source	SGAB001	SGAB010	SGAB013
M. mycoides subsp. *mycoides* Small Colony	Shawawa	L. Prozesky[a]	19.6 (0.2)[b]	22.2 (0.6)	22.8 (0.4)
	Ondangwa	L. Prozesky	19.3 (0.1)	20.2 (0.2)	21.6 (0.6)
	PG1	APHIS[c]	19.0 (0.1)	21.3 (0.8)	21.4 (0.2)
	Cameroon	APHIS	21.8 (0.2)	26.8 (2.0)	25.6 (0.5)
	Gladysdale	APHIS	22.8 (0.3)	22.4 (0.2)	24.0 (0.4)
M. mycoides subsp. *mycoides* Large Colony	GM12	S.J. Geary[d]	ND[e]	ND	ND
M. mycoides subsp. *capri*	PG3	S.J. Geary	ND	ND	ND
M. capricolum subsp. *capricolum*	California kid	S.J. Geary	ND	ND	ND
M. sp. bovine group 7	PG50	S.J. Geary	ND	ND	ND
M. bovis	Donetta	S.J. Geary	ND	ND	ND
M. dispar	ATCC 27140	S.J. Geary	ND	ND	ND

[a] Leon Prozesky, Department of Paraclinical Sciences, University of Pretoria, Onderstepoort, South Africa.
[b] Mean C_T value of three data points; standard errors of the means are shown in parentheses.
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[e] Not detected.

(Bashiruddin et al., 2005). Le Grand et al. (2004) assessed several PCR tests for identification of species belonging to the *M. mycoides* cluster and reported that two tests were needed to confirm the identification of *M. mycoides* SC strains. Despite the existence of numerous assays for *M. mycoides* SC, these reports support the need for improved diagnostic capabilities for CBPP. The aim of this research was to design and develop a rapid diagnostic assay for the detection of *M. mycoides* SC. We describe the development of three TaqMan real-time PCR assays for the specific detection of *M. mycoides* SC.

2. Materials and methods

2.1. Strains and growth conditions

The mycoplasma species and strains listed in Table 1 were used in this study. *M. mycoides* subsp. *mycoides* SC strains Shawawa and Ondangwa were obtained from Professor Leon Prozesky (University of Pretoria, Onderstepoort, South Africa). *M. mycoides* subsp. *mycoides* SC strains PG1, Cameroon, and Gladysdale were obtained from the USDA Animal and Plant Health Inspection Service (APHIS), Plum Island Animal Disease Center, Greenport, NY, USA. *M. mycoides* subsp. *mycoides* Large Colony strain GM12, *M. capricolum* subsp. *capricolum* strain California kid, *M. mycoides* subsp. *capri* strain PG3, *Mycoplasma* sp. bovine serogroup 7 strain PG50, *Mycoplasma bovis* strain Donetta, and *Mycoplasma dispar* ATCC 27140 were obtained from the mycoplasma collection at the University of Connecticut. All strains were grown in Fortified Commercial medium (Foy et al., 1979).

2.2. Clinical samples

Bronchoalveolar lavage (BAL) samples were obtained from cattle experimentally infected with *M. mycoides* SC. Five animals (#'s 29, 31, 32, 34, and 35) were inoculated endobronchially with approximately 10^{10} viable cells of *M. mycoides* SC strain Gladysdale. BAL was performed on each animal both prior to inoculation and 8 days post-inoculation using a bronchoscope and 120 ml sterile physiologic saline (0.9% NaCl). For each sampling, 1 ml of bronchoalveolar lavage fluid was centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatants were removed and the pelleted materials were resuspended in 500 μl phosphate buffered saline. The samples were divided equally into two tubes; one portion was boiled for 10 min and the other was used for DNA extraction. Animal experiments were conducted under federal guidelines and the USDA, Plum Island Animal Disease Center policies on animal care and use.

2.3. DNA extraction

Mycoplasma genomic DNA from 25 ml cultures and total DNA from BAL samples were prepared using the Easy DNA kit (Invitrogen, Carlsbad, California) according to the manufacturer's instructions.

2.4. Genetic targets

Three genetic targets were identified for development of *M. mycoides* SC-specific TaqMan probes. Oligonucleotide sequences for probe SGAB001 and its respective primers (Table 2) were designed based on recommendations made in the TaqMan Universal PCR Master Mix protocol and a consensus alignment of *M. mycoides* cluster 16S rRNA sequences retrieved

Table 2
Oligonucleotide primers and probes used for the real-time PCR detection of *M. mycoides* SC

Name	Sequence
SG1098	5′-ATG GAC GAA AGT CTG ATG AAG CAA TGC-3′
SG1099	5′-TCT GGT AAG GTA CTG TCA AGA TAA AGT CAT-3′
SGAB001	5′-6FAM ACA ACA GAG ATT TAC AAC MGBNFQ-3′
SG1352	5′-GTT TTT TAG GAA TTT TGT AAA TAG CTC AAT T-3′
SG1353	5′-AGT GAA GTT TCT AAA TCA ATC CAA TCA G-3′
SGAB010	5′-6FAM TCA ATT AGA CTG TAA TGA AAT AT MGBNFQ-3′
SG1358	5′-AAT CAC CCA AAT TAG ATA GAC CTA GTT CA-3′
SG1359	5′-GGT TAC TGA GCA GCT GAG GAA GA-3′
SGAB013	5′-6FAM TCA ATT TCT CCA GTT TTA GCA AA MGBNFQ-3′

6FAM, fluorescent dye 6-carboxyfluorescein; MGBNFQ, minor groove binder/non-fluorescent quencher.

from GenBank. Probes SGAB010 and SGAB013 were identified as candidate *M. mycoides* SC-specific genetic targets through random sequencing of genomic DNA from *M. mycoides* SC strains Shawawa and Ondangwa. Random DNA fragments were generated by incomplete digestion of genomic DNA with *Tsp*509I restriction endonuclease (New England BioLabs, Beverly, Massachusetts). Fragments larger then 1.0 kb were size selected using CHROMA SPIN + TE-1000 columns (BD Biosciences, San Jose, California) and cloned into *Eco*RI digested and dephosphorylated pUC19 plasmid (New England BioLabs). Plasmid DNA was grown in *Escherichia coli* One Shot Max Efficiency DH5α T1 competent cells (Invitrogen) and extracted using PerfectPrep Plasmid 96 Vac Direct Bind (Eppendorf, Westbury, New York). Sequencing reactions were performed with M13 forward and reverse primers using Applied Biosystems (ABI) BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and run on an ABI 3730xl DNA Analyzer (Applied Biosystems). ABI Data Sequencing Analysis Software v5.2, Phred (Ewing et al., 1998), Phrap (Ewing and Green, 1998), and CAP3 (Huang and Madan, 1999) were used for data collection and analysis. The resulting contigs were compared to the GenBank database using BLAST (Altschul et al., 1990). Based on this analysis, two contigs were selected as candidate *M. mycoides* SC-specific sequences. Probes SGAB010 and SGAB013 and respective primer sequences (Table 2) were selected using Primer Express software (Applied Biosystems).

TaqMan probes were synthesized (Applied Biosystems, Table 2) to include the fluorescent dye 6-carboxyfluorescein (6FAM) on the 5′-end and minor groove binder/non-fluorescent quencher (MGBNFQ) on the 3′-end. The oligonucleotide primers used for amplification are listed in Table 2 (MWG Biotech, High Point, North Carolina).

The nucleotide positions described throughout this manuscript refer to the complete genome sequence of *M. mycoides* SC strain PG1 reported by Westberg et al. (2004) (GenBank accession # BX292980).

2.5. Real-time PCR

PCR amplification and real-time detection on reaction mixtures, containing known concentrations or 1 μl of BAL sample as template DNA, 0.9 μM concentration of each forward and reverse primer, 0.1 μM concentration of probe, and TaqMan Universal PCR Master Mix (Applied Biosystems) in a total volume of 20 μl, were performed with the SmartCycler System (Cepheid, Sunnyvale, California). Reaction conditions included 2 min at 50 °C followed by 10 min at 95 °C and then 40 cycles of 15 s at 94 °C and 60 s at 60 °C. The default threshold limit was set at 30 and the optics detection was turned on for the cycle steps at 60 °C. The data were exported to Microsoft Excel for analysis.

3. Results

3.1. Genetic targets

Probe SGAB001 targets a unique polymorphism of the *M. mycoides* SC 16S rRNA gene *rrn*A (Pettersson et al., 1996). Probe SGAB010 targets a hypothetical lipoprotein (MSC_0500; GenBank accession # BX292980) and probe SGAB013 targets a hypothetical CDS (MSC_0198; GenBank accession # BX292980).

3.2. Standard curve and detection limit

Serial 10-fold dilutions of *M. mycoides* SC genomic DNA and corresponding C_T values were used to plot standard curves for each probe (Fig. 1). An inverse linear relationship was observed between the C_T values and target concentration over seven orders of magnitude for probe SGAB001 and over six orders of magnitude for probes SGAB010 and SGAB013. For probes SGAB001, SGAB010, and SGAB013 curve slopes were −3.666, −4.047, and −4.253 and linear square regression coefficients were 0.9998, 0.9959, and 0.9957, respectively. The limits of detection were 100 fg of genomic DNA for probe SGAB001 and 1 pg of genomic DNA for probes SGAB010 and SGAB013. Based on the genome size of *M. mycoides* SC strain PG1 (Westberg et al., 2004), these limits of detection correspond to the genomic equivalent of approximately 10^2 and 10^3 organisms, respectively.

3.3. Specificity

TaqMan probe specificity was tested against DNA from 11 mycoplasma strains, including five *M.*

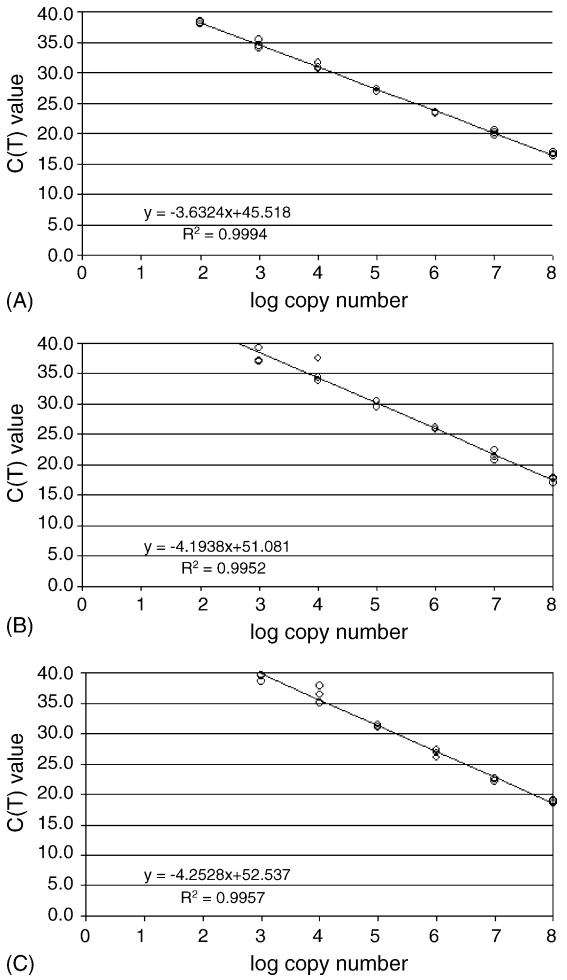

Fig. 1. Standard curves generated by the analysis of known amounts of *M. mycoides* subspecies *mycoides* Small Colony genomic DNA with probes (A) SGAB001, (B) SGAB010, and (C) SGAB013. Three data sets were used to generate each standard curve. The linear regression lines calculated using the mean C_T values for each dilution of genomic DNA are shown. The coefficient of correlation for each standard curve is greater then 0.995.

mycoides SC strains, four additional strains belonging to the *M. mycoides* cluster, and two strains of bovine mycoplasma species not belonging to the *M. mycoides* cluster (Table 1). Only the five strains of *M. mycoides* SC gave specific signal, with 10 ng of genomic DNA yielding mean C_T values of 20.5, 22.6, and 23.1 with probes SGAB001, SGAB010, and SGAB013, respectively.

3.4. Analysis of clinical samples

All five animals inoculated with *M. mycoides* SC strain Gladysdale exhibited elevated temperatures. Two animals (#29 and #31) were euthanized at days 10 and 13 post-inoculation, respectively, due to the severity of clinical signs consistent with CBPP. In addition to elevated temperature, these animals had dyspnea, tachypnea, mucoid nasal discharge, pleurodynia, inappetence, were lethargic, and stood with abducted elbows. The three other animals (#'s 32, 34, and 35) appeared to recover clinically and were euthanized at 37 days post inoculation. All five animals exhibited pulmonary lesions consistent with CBPP upon necropsy.

All BAL samples obtained from the experimentally infected cattle were positive by real-time PCR using any of the three probes (SGAB001, SGAB010, and SGAB013) and 1 μl of either boiled or DNA extracted template preparation (Table 3). Pre-inoculation samples were negative for *M. mycoides* SC with probes SGAB001, SGAB010, and SGAB013 (Table 3).

4. Discussion

M. mycoides subsp. *mycoides* Small Colony is classified by the USDA as a high consequence livestock pathogen. Although the United States has been free of CBPP for over a century, the potential economic impact of this disease on the cattle industry necessitates the development of rapid, sensitive, and specific diagnostic assays for the detection of *M. mycoides* SC. Here we present data generated during development of three TaqMan-based, real-time PCR assays for the specific detection of *M. mycoides* SC, assays providing several advantages over currently available CBPP diagnostic assays.

TaqMan real-time technology (Applied Biosystems, Foster City, California) has revolutionized DNA-based diagnostic procedures utilizing fluorogenic oligonucleotide probes to enable the detection of specific PCR products throughout the amplification process. Intact TaqMan MGB probes contain a reporter fluorescent dye on the 5′-end of the oligonucleotide and a non-fluorescent quencher dye on the 3′-end. They anneal to specific target sequences within the PCR amplicon and are hydrolyzed by the 5′-exonuclease activity of *Taq* DNA polymerase during amplification. The cleavage of

Table 3
Results of real-time PCR for three *M. mycoides* SC-specific TaqMan probes on bronchoalveolar lavage samples from cattle pre-inoculation (p.i.) and at 8 days (8d) post-inoculation

Animal ID#	Sample preparation	SGAB001		SGAB010		SGAB013	
		p.i.	8d	p.i.	8d	p.i.	8d
29	Boiled	−[a]	+	−	+	−	+
	Extracted	−	+	−	+	−	+
31	Boiled	−	+	−	+	−	+
	Extracted	−	+	−	+	−	+
32	Boiled	−	+	−	+	−	+
	Extracted	−	+	−	+	−	+
34	Boiled	−	+	−	+	−	+
	Extracted	−	+	−	+	−	+
35	Boiled	−	+	−	+	−	+
	Extracted	−	+	−	+	−	+

[a] (−) negative, not detected; (+) positive, $C_T < 40$.

the probe separates the reporter dye from the quencher dye, resulting in increased fluorescent signal. Increases in fluorescent signal from unquenched reporter dye is proportional to specific amplification and measured in real-time throughout the course of the reaction, greatly decreasing the complexity and time required to conduct the PCR assay. Positive TaqMan reactions are characterized by the point during cycling at which signal is detected above baseline fluorescence, referred to as the threshold cycle (C_T) value, simplifying interpretation of assay results and providing a quantitative measure of template concentration. In addition, the presence of a minor groove binder at the 3′-end of TaqMan MGB probes increases the melting temperature of the probe, enabling more accurate discrimination of the target sequence.

This format has several advantages over assays currently available for the detection of *M. mycoides* SC. As a PCR-based assay, it is not dependent on the direct cultivation of the causative agent, allowing for more rapid results. TaqMan assays, including sample preparation, can be performed in under 2 h and require no post-amplification manipulation and analysis of the PCR products, such as restriction endonuclease or sequence analysis. TaqMan MGB probes are highly specific and recommended for the discrimination of single nucleotide differences. Each assay can be performed in a single closed tube, greatly reducing hands-on manipulations and the likelihood of cross contamination, or alternatively, can be performed in a 96-well format for high throughput analysis of samples. Finally, the Cepheid SmartCycler is a portable system that can be utilized in the field for pen-side testing of animals.

Our data demonstrate that the TaqMan probes SGAB001, SGAB010, and SGAB013 reliably detect multiple strains of *M. mycoides* SC and are specific, failing to detect related members of the *M. mycoides* cluster. Each of these probes detected *M. mycoides* SC in bronchoalveolar lavage samples obtained from experimentally infected animals. Both boiled lysis preparations and purified DNA preparations were assessed and were found to yield positive results, indicating that any PCR inhibitors that might have been present in the clinical samples did not affect the results of these assays.

Although probe SGAB001 targets a unique single nucleotide polymorphism in the *M. mycoides* SC 16S rRNA gene *rrn*A, probes SGAB010 and SGAB013 target *M. mycoides* SC-specific open reading frames (ORF) (MSC_0500 and MSC_0198, respectively). These ORFs are predicted to encode a lipoprotein (MSC_0500) and a transmembrane protein (MSC_0198) (Westberg et al., 2004). Previously described *M. mycoides* SC PCR-based assays have also targeted surface proteins. A nested PCR method targeted a region overlapping two genes (MSC_0013 and MSC_0014) that encode lipoproteins (Miserez et al., 1997). MSC_0014 encodes for a 72 kDa immunodominant protein (Cheng et al., 1996). The PCR assay described by Dedieu et al. (1994) targets a region overlapping with the 3′-end of MSC_0390, which encodes the variable lipoprotein Vmm (Persson et al., 2002). These data might be an indication of the importance of *M.*

mycoides SC surface molecules in host-specificity and virulence and are logical targets for further investigation into mechanisms of pathogenicity and survival of *M. mycoides* SC. In addition, *M. mycoides* SC-specific proteins are potential candidate targets for the development of improved vaccines.

Early recognition of *M. mycoides* SC is essential for effective CBPP control and is critical for biosurveillance in the United States. In addition, an easy-to-use, portable diagnostic test would be beneficial for local veterinary staff to facilitate herd screening in efforts to control and prevent outbreaks of CBPP in many African countries where CBPP is endemic. With the appropriate validation experiments outlined by APHIS and the OIE, the TaqMan-based real-time PCR assays described in this paper will provide veterinarians and diagnostic laboratories worldwide with a rapid, specific, and sensitive test for the detection of *M. mycoides* SC.

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